Insm1: Orchestrating cellular mimicry in the thymus medulla

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The thymus is the primary production site for a functional and self-tolerant T-cell repertoire. The establishment of immune tolerance is mediated by the generation and presentation of tissue-restricted self-antigens (TRAs) by medullary thymic epithelial cells (mTECs), which screen T-cell receptors for self-reactivity to eliminate autoreactive T cells. Recent studies have extended our understanding of mTEC heterogeneity by examining the mechanisms by which mTECs mimic the peripheral self[1]. Several studies have shown that mTECs use lineage-defining transcription factors normally expressed extrathymically to generate intrathymic cellular mimics of diverse peripheral cell identities[2-4]. These novel subsets of unconventional mTECs were collectively termed “mimetic mTECs”[3]. These mTECs mimic the transcriptional profile and morphology of well-defined, highly specialized cell types, such as endocrine cells, microfold cells and tuft cells, throughout the body, but importantly, they still retain an mTEC identity rather than being converted to a bona fide peripheral cell type[5]. Functionally, thymic mimetic subsets have nonredundant roles in thymus function, for example, in the induction of antigen-specific tolerance and the regulation of thymic involution[2, 3].

A recent focus has been the analysis of a specific mimetic mTEC subset that mimics epithelial neuroendocrine cells initially named endoTECs by Givony et al. Insulinoma-associated protein 1 (Insm1) was identified as a lineage-defining master regulator of these cells. Most recently, Tao et al. investigated the importance of Insm1 in mTEC mimetic development, confirming its role as a lineage-defining transcription factor for endoTECs, which they termed neuroendocrine mimetic cells. Furthermore, they identified an essential role for Insm1 as a regulator of Aire^{+} mTEC development and self-antigen expression.

Using a reporter mouse model and immunofluorescence analysis, Tao et al. reported that endogenous Insm1 protein is expressed not only by neuroendocrine mimetic cells but also by upstream transit-amplifying cells (TACs) and Aire-expressing mTECs. In adult *Insm1-thycKO* mice, TEC-specific loss of *Insm1* resulted in a significant reduction in Aire protein expression per cell and a reduction in the overall frequency of Aire-expressing mTECs. The upstream role of Insm1 in Aire^{+} mTEC development was further determined using Insm1-overexpressing mice, which exhibited increased Aire-expressing mTECs and an expanded neuroendocrine mimetic cell population. Interestingly, Givony et al. observed no differences in the mRNA expression of *Aire* in mTECs II isolated from *Insm1-thycKO* mice, highlighting discrepancies between *Aire mRNA* and Aire protein levels and identifying a need to resolve this disparity. Analysis of TRA expression in *Insm1-thycKO* mice revealed that both Aire-dependent and Aire-independent TRA expression were significantly altered, indicating that Insm1 regulates tolerance induction through not only neuroendocrine-associated TRAs but also Aire[(4)]. This cooperation of Insm1 and Aire was also evident in Insm1-overexpressing mice,

in which TRA expression was upregulated[4]. The authors compared the chromatin binding sites of Insm1 and Aire and found that the majority of Insm1 binding sites were cooccupied by Aire in superenhancer regions[4]. Moreover, they demonstrated that Insm1 can bind to the Aire promoter region and distal regulatory sequences, suggesting a possible mechanism by which Insm1 can control *Aire* expression[4].

Insm1-thycKO mice exhibited impaired development of other mimetic mTEC populations. Previously, Givony et al. reported an increase in microfoldTEC-specific gene expression in the absence of *Insm1*, while Tao et al. reported reduced enterohepato mimetic TECs, which do not express *Insm1*. This is likely a consequence of multiple mimetic TECs being developmentally derived from Insm1- and Aire-expressing mTECs II. Aire is believed to control the expression of lineage-defining transcription factors in extrathymic mimetic TECs and thus may be required for the accumulation of some types of mimetic cells[3]. Thus, the direct effect of Insm1 deficiency on Aire expression may result in alterations in downstream mimetic mTEC populations. Alternatively, Insm1 may have a direct effect on the differentiation of mimetic subsets independent of its effects on Aire expression. Overall, these findings suggest that Insm1 not only is a pioneering transcription factor critical for neuroendocrine mimetic cell differentiation but also plays important roles in Aire-expressing mTECs and other mimetic TEC subsets.

To understand the effects of Insm1 on the establishment of tolerance, multiple alterations in TEC heterogeneity caused by Insm1 depletion must be considered. Although infiltrates were detected in organs enriched with endocrine cells,

autoimmune reactions in tissues typically associated with Aire deficiency were also observed. Importantly, not all Aire-associated tissues were targeted. For example, there were no reactions to eye-specific antigens, which are the primary targets in Aire-deficient mice on a Black-6 background[6]. Additionally, tissues not associated with Aire deficiency, such as brown adipose tissue, were targeted. Taken together, these results indicate that Insm1 deficiency in TECs results in widespread autoimmunity, which can include but is not exclusively a consequence of alterations to Aire-expressing mTECs. Furthermore, Tao et al. reported that regulatory T-cell development was impaired in *Insm1-thycKO* mice, suggesting that the autoimmune defects observed in these mice could be further exacerbated by a breakdown in peripheral tolerance mechanisms.

Moreover, Insm1-dependent neuroendocrine mimetic cells have been identified as the primary source of intrathymic ghrelin, which is known to play a role in modulating age-dependent thymic involution. Assessment of 15- to 16-week-old *Insm1-thycKO* mice revealed significantly reduced thymic cellularity that could be rescued by intrathymic injection of ghrelin, suggesting that neuroendocrine mimetic cells are novel modulators of thymic involution[2]. Thus, mimetic TECs not only play a role in the establishment of tolerance but can also mediate homeostatic processes such as thymic involution, which remains poorly understood at the mechanistic level. The use of spatial and transcriptomic approaches on human TEC subsets have revealed comparable heterogeneous mimetic-like populations in the human thymus[7]. These observations highlight the importance of a better understanding of mimetic populations, as the manipulation of these cells may have therapeutic potential for

treating dysregulated immune disorders and ameliorating the effects of aging. Furthermore, Insm1 has been shown to be highly expressed by mTECs in thymic squamous cell carcinoma, further indicating the need to understand the regulation and function of Insm1 in TECs[8].

Recent advances in mimetic mTECs, including observations by Tao and colleagues, reveal the gaps in our knowledge about how this highly specialized set of cells regulates tolerance. If loss of Insm1-dependent mimetic cells leads to autoreactivity bias to certain endocrine-rich tissues, does this suggest that other mimetic populations also control the establishment of immune tolerance to the specific tissue they resemble? If so, how are the mimetic mTEC subsets represented within the thymus determined? The differentiation of intrathymic T-regulatory cells and other nonconventional T-cell subsets is dependent upon mTEC interactions[9]. Is it possible that interactions with specific mimetic mTECs influence the divergence of T-cell lineages and perhaps even determine the tissue to which they home?

Detailed atlases of the TEC compartment for cell transcriptional states and chromatin landscapes have given us a fresh appreciation of the complexity of the role of mTECs in the establishment of tolerance. Tao et al. confirmed an essential role for Insm1 in neuroendocrine mTEC development and identified a novel function for this transcription factor in the regulation of Aire expression. Although early studies reported histological and morphological analysis of epithelial heterogeneity in the thymus medulla[10], a clearer understanding of the molecular regulators of mimetic cells and the functional relevance of these cells represents an important emerging field within the study of the thymus. Furthermore, while the requirement for Aire in

self-antigen expression has been known for more than 20 years[6], most of the underlying mechanisms of the functions of Aire are still unknown. Mimetic cells have contributed to the understanding of how Aire influences TRA expression and tolerance induction, and a better understanding of these newly understood cell types will provide many more insights into the mechanisms of Aire functions. We are only just beginning to unravel the complexity of self-presentation in the medulla and its influence on T-cell development.

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158 Figure legends

159 Figure 1. Insm1 is an essential lineage-defining transcription factor involved in
160 neuroendocrine mimetic cell development and Aire expression. **A.** In the steady-state
161 adult thymus, Insm1 is expressed by multiple mTEC subsets. **B.** In the absence of
162 Insm1, Aire expression in mTECs is significantly reduced, and the neuroendocrine
163 mimetic mTECs are absent. As a result, tissue-restricted antigen expression in the
164 thymus is significantly impaired.

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Adult Thymus